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Immune System Responses to Ectoparasite Infections in Nestling Barn Swallows *Hirundo
rustica*: an Experimental Approach

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Glossary

Acquired immune response: general term used to describe a specific immune response; able to recognize a threat; associated with lymphocytes

Altricial: offspring that are incapable of departing from the nest after hatching; dependent on parental care; relatively undeveloped upon hatching

Antibody: immunoglobulin; protein produced by lymphocytes; used to identify and neutralize foreign objects

Antigen: substance that induces the production of antibodies; may be a foreign substance from the environment or formed within the body

Ectoparasite: a parasite that lives on the exterior of an organism such as on the skin or feathers

Endoparasite: a parasite that lives on the interior of an organism such as in the gut or bloodstream

Heterophil: granulocytic white blood cell predominately associated with the innate immune response; one of the most numerous and functionally significant white blood cells in avian species

Innate immune response: general term used to describe a non-specific immune response; does not specifically recognize a threat; first line of defense against a pathogen; associated with heterophils

Leukocyte: white blood cell

Lymphocyte: white blood cell associated with the acquired immune system; one of the most numerous and functionally significant in avian species

Pathogen: infectious agent

Precocial: offspring that are capable of leaving the nest upon hatching; not entirely dependent on parental care; more developed upon hatching than altricial nestlings

Abstract

In altricial [helpless] birds, ectoparasite [external parasite] exposure during development may elicit a costly immune response requiring nestlings to redistribute energy away from growth and development and towards parasite defense. That altricial nestlings are born with an immature immune system may have implications for the mechanisms they use for immune defense. I studied how nestlings defend themselves against ectoparasites as well as how nestling immune defense interacts with nestling mass. I specifically examined how innate versus acquired immune responses react to ectoparasite exposure by assessing leukocyte [white blood cell] proportions as well as how the different branches of the immune system interacted with nestling mass. Nestlings experimentally exposed to parasites during development tended to have higher proportions of heterophils [innate leukocyte] and lower proportions of lymphocytes [acquired leukocyte] compared to nestlings not exposed to ectoparasites. When exposed to ectoparasites, the acquired immune system response required a greater body mass than the innate immune system response; nestlings with higher body mass exhibited a higher proportion of lymphocytes and had a lower proportion of heterophils. My findings aid in the understanding of how altricial nestlings mount an immune response to ectoparasites as well as lay a foundation for future studies exploring how the costs of defense against ectoparasite may interact with somatic [the body's] growth and immune system development.

1. Introduction

Life history theory suggests that organisms distribute resources between multiple energetically demanding activities in a way that optimizes survival and reproduction (Saino et al. 1998, Cain et al. 2008). According to the life history theory, nestlings that are confined to a nest during development must divide resources between somatic growth and investment in processes that reduce their proximate mortality risk during the nesting period, such as parasite defense and predator avoidance (Stambaugh et al. 2011). Therefore, exposure to parasites during early stages of life may be an important environmental factor influencing development (Reed et al. 2012). In particular, ectoparasite defense has been hypothesized to limit the resources available to a nestling for somatic growth because it may require an energy-consuming immune response (Blanco et al. 2001, Brommer et al. 2011). Nestling immune response is also thought to be comprised of both the innate and acquired immune system (see Table 1); the two branches of the immune system are, furthermore, thought to involve different costs and may respond differently to a parasitic infection (Norris and Evans 2000). Little is known about the development of the immune system in nestlings; more research is needed to enhance the understanding of how the undeveloped immune system responds to parasite exposure is needed (De Coster et al. 2010).

Overall, the exact immune response to parasites in free-living nestlings and the interaction between particular immune responses and body size is poorly understood. While the degree to which ectoparasites may influence nestling body size and growth has been studied the complexity of the nestling immune system and its specific function in parasite defense is unknown (Zuk and Stoehr 2002, Brommer et al. 2011). Studies examining the immature immune system of nestlings have mainly done so in laboratory experiments using unnatural antigens

[foreign substance] to trigger an immune response (Norris and Evans 2000, Parejo et al. 2007, Palacios et al. 2009). The influence of parasites on body size remains to be integrated with investigation of the immature immune response of nestlings to a natural immune challenge. To better understand the influence of ectoparasite exposure on development, research must focus on how the different components of the nestling immune system (innate vs. acquired) respond to ectoparasite exposure as well as how each component and overall immune response interacts with body size. Songbirds with altricial nestlings are a suitable system for exploring the consequences of parasite exposure because both physiological and determinant morphological growth occur in a discrete location allowing for good experimental control over the environment in which nestling development occurs (Brommer et al. 2011).

Here, I examine the influence of ectoparasite exposure during nestling development on investment patterns of immune response (innate vs. acquired), including an analysis of different aspects of the immune defense to ectoparasites and the potential trade-off between immune defense and body size at a standardized time during development as a proxy for somatic growth (Table 2). I measured leukocyte proportions (a measure of immune response) in relation to parasitism and body size to address the following questions: (1) Do ectoparasites elicit an immune response in nestlings? After finding out that they do, I addressed (2) whether there are differences in the innate and acquired immune system by examining heterophil [innate leukocyte] and lymphocyte [acquired leukocyte] proportions in nestlings. Finally, I wanted to begin to investigate how ectoparasite exposure may influence the immune system and somatic development by asking (3) whether ectoparasite exposure influences the relationship between the immune response of nestlings and their mass at a standardized time during early development.

2. Background

2.1 Nestling development and parasitism

Using nestling development as a model system, researchers have come to understand that parasite exposure at an early age can influence somatic growth (Table 2). At the most severe level, parasitism has been found to terminate nestling development and reduce survival (Merino et al. 2001, Dudaniec et al. 2006). However, nestlings are believed to avoid mortality by reallocating energy from used for growth towards parasite defense (Saino et al. 1998, Zuk and Stoehr 2002). Furthermore, studies of altricial nestlings have found that nestlings may invest more energy in wing and feather growth, features of morphology critical for flight out of the nest (Saino et al. 1998, Szép and Møller 1999). Yet, the development of flight-related morphology may be at the detriment of developing other traits, including body mass and tarsus length (Saino et al. 1998, Szép and Møller 1999). The above adjustments in nestling development are though to shorten the duration of the nesting period in order to limit parasite exposure (Møller 1990, Saino et al. 1998). In particular, nestlings subjected to parasite exposure were of smaller mass and tarsus length than their non-parasitized counterparts presumably in order to reallocate energy towards the energetically costly immune defense (Hörak et al. 1999, Szép and Møller 1999, Merino et al. 2001, Lobato et al. 2005). The trade-off believed to occur between immune response and nestling body size is further emphasized by the ability of nestlings with higher resource availability (i.e. larger mass and body size) to mount a stronger immune defense against an immune challenge than those with more limited resources (Hörak et al. 1999).

However, when examining energy trade-offs in response to parasitism one key variable has been largely ignored: how the innate versus acquired branch of the immune system respond to immune challenges during early development (Table 1). The immune system of the nestling is

believed to be poorly developed upon hatching and may take weeks to fully mature (De Coster et al. 2010). Therefore, nestlings exposed to parasites may not only be balancing resources between immune defense and morphological growth but also could be limited in their ability to respond because of an immature immune system (Saino et al. 1997, Brommer et al. 2011). The relationship between ectoparasites and the immature immune system as well as the influence parasite exposure has on the interaction between somatic growth and nestling immune defense remains unresolved.

2.2 The avian immune system response to ectoparasites

While not all ectoparasite infections solicit an immune response, the immunological defense of adult birds to ectoparasites is commonly an inflammatory response (Møller and Rózsa 2005, Mazur et al. 2007, Owen et al. 2010). Ectoparasite exposure may also elicit an antibody driven response involving proteins produced by lymphocytes that recognize and neutralize foreign invaders (Owen et al. 2010). Overall, immune system defense against ectoparasites is believed to rely on both branches of the immune system (Table 1, Owen et al. 2010). Characteristics of the innate immune system make it a vital protection mechanism in the initial stages of an infection (Table 1, Masello et al. 2009). Whereas characteristics of the acquired immune system make it essential for long-term immunity (Table 1, Zuk and Stoehr 2002, Bonneaud et al. 2003). During an inflammatory response, granulocytes [non-specific response] migrate into the circulating blood, engulf antigens [foreign substance] and trigger a lymphocyte-mediated specific response, which recruits more granulocytic cells into the epidermis [outer layer of skin] causing the skin to swell (Owen et al. 2010). This reaction inhibits the ectoparasite from continuing to feed and reproduce on its host (Owen et al. 2009).

The response of the immune system of nestlings to ectoparasites is poorly understood. Based on data mostly gathered in a laboratory setting, it is believed that the acquired immune system can take as long as weeks to months to fully mature (Sindik and Lill 2009). For example, tree swallow nestlings do not reach adult proportions of lymphocytes until 18 days after hatching (Palacios et al. 2009). Because the acquired immune system generates a specific response, it must develop lymphocytes able to recognize a broad range of antigens; this process is thought to be expensive (Klasing and Leschinsky 1999). The innate immune system is thought to be less costly to develop and more readily available; which is why nestlings may have to rely more on their innate immune system as a primary mechanism of defense (Lee 2006, Masello et al. 2009). Moreover, recent studies indicate that the difference between adult and juvenile immune response is reflected by the proportion of innate versus acquired immune response; as nestlings develop, they shift away from a strong dependence on their innate immune system and better utilize their acquired immune system (Palacios et al. 2009). Furthermore, heterophils proliferate in nestlings in response to blood-sucking parasites, indicating a stronger investment in innate immune function in response to ectoparasites during the developmental stage (Merino et al. 2001, Szabo et al. 2002, Lobato et al. 2005). However, more studies must be conducted in wild populations with natural parasites to understand how the nestling's immune system reacts to an ectoparasite.

2.3 Measuring the immune response during nestling development

Leukocytes [white blood cells] constitute the basis of the immune system (Sindik and Lill 2009). The relative proportion of leukocyte types is referred to as a leukocyte profile or white blood cell differential (Table 1, Davis et al. 2004). The proportions of leukocyte types have been used to assess infection status as well as general patterns of the immune system in avian species

(Davis et al. 2004, Vinkler et al. 2010). Heterophils and lymphocytes are the most numerous and functionally significant leukocytes in avian blood, making them a useful tool to analyze immune responses (Sindik and Lill 2009, Wojczulanis-Jakubas et al. 2012). Most commonly, white blood cell differentials have been used to calculate heterophil to lymphocyte ratios (H/L ratio, hereafter). Higher H/L ratios have been found to be positively correlated with parasite levels and have been linked to other environmental stressors (Lobato et al. 2011, Müller et al. 2011). The H/L ratio has also been related to specific components of the immune system (Davis et al. 2004). Heterophils can serve as an indirect measure of innate immune function whereas lymphocytes can serve as an indirect measure of the acquired immune function (Davis et al. 2004, Owen and Moore 2006, Dehnhard et al. 2011). Investigating the individual proportions of heterophils and lymphocytes can be used to examine questions pertaining to the different branches of immune response, which is particularly relevant for exploring the immune system response to parasite exposure during early development (Quillfeldt et al. 2008, Parejo and Silva 2009, Dehnhard et al. 2011).

3. The study system

*3.1 Study system overview - the barn swallow, *Hirundo rustica erythrogaster**

The North American barn swallow, *Hirundo rustica erythrogaster*, is a socially monogamous, migratory songbird (Brown and Brown 1999). A typical adult lifespan ranges from 0 – 7 years, with an average of 2 years (Brown and Brown 1999). Whereas adult survival is quite high (on average 55%), nestling survival is quite low (on average 30%) due to mortality on the natal territory as well as during the first year of life (Hubbard unpublished). Based upon previous studies on links between avian body condition and migration success, parasitic infection

may be an important factor in high mortality rates of nestlings (Legge 2002, Monticelli and Ramos 2012).

Barn swallow nestlings develop over a two-week period in a discrete nest location where they are provided parental care before becoming fully independent. Upon hatching, altricial nestlings, such as barn swallows, are believed to have less mature immune systems than nestlings that are precocial [relatively developed] due to shorter periods of egg incubation and, therefore, shorter time for immune system development prior to hatching (Ricklefs 1992). Altricial nestlings are also believed to be prone to higher levels of ectoparasite exposure due to limited mobility, making the questions of ectoparasite influence on immune response and body size of particular importance (De Coster et al. 2010). Furthermore, limited nest mobility gives researchers a great deal of control over parasite exposure during nestling development.

3.2 Study system overview - the northern fowl mite, Ornithonyssus sylviarum

Mites are a diverse group of arthropods belonging to the subclass Acari; at least 2500 species have been found to be associated with birds (Proctor and Owens 2000). Even though mites are a part of many ecological systems, the cost of mites on their host and how mites may impact host life history remains unresolved.

The northern fowl mite, *Ornithonyssus sylviarum*, is a common blood-sucking arthropod found mainly on small passerine birds (Owen et al. 2009). The northern fowl mite is one of the dominant parasites of barn swallow nestlings; mites can be found living in the nest and feeding on nestlings (Hund et al. unpub). Mites, generally, rapidly reproduce and are capable of building up extremely high population densities quickly (Proctor and Owens 2000). At high levels of infection in poultry, the northern fowl mite has been shown to cause anemia, emaciation and immune shock due to blood loss and bite wounds (Furman 1963).

In general, most work examining parasites and their consequences have been focused on endoparasites [internal parasites]. Ectoparasites can generate a variety of responses in developing nestlings (Table 2); the specific relationships between mites, defense, and potential tradeoffs in juveniles still remain unclear.

4. Methods

During the summer of 2012, a large field study focusing on parasites and sexual selection in the North American barn swallow *Hirundo rustica erythrogaster* was conducted across 42 sites in Boulder County, Colorado between April and September 2012.

4.1 Cross-fostering experiment

Only cross-fosters involved in the second half of the field season were involved in the parasite-manipulation experiment. For the partial reciprocal cross-foster experiment (n=10 parasite nest pairs; 20 experimental nests; 15 unpaired control nests: 157 nestlings: n= 6 natural nest pairs; 12 experimental nests; 6 unpaired controls nests, 47 nestlings) nests were paired across field sites based on hatch day (day 0) and number of nestlings in each nest (4-5). On day 2, nestlings with the smallest and largest mass, respectively, were exchanged between paired nests maintaining the natural sibling hierarchy of each nest. During the exchange of nestlings on day 2, if nests were a part of the parasite treatment, each nest was sterilized after nestlings were removed using an industrial heat gun and an infrared laser thermometer to heat the nest to 125°C in order to kill all mites in the nest. 75 field-collected blood-feeding mites were then added to each nest after it had returned to room temperature. This allowed control for the initial parasite exposure level in each nest. For nestlings involved in the natural cross-foster treatment, any parasites present were counted but left as is. The legs of the exchanged foster nestlings were marked with nontoxic green marker in order to keep track of which ones were cross-fostered into

the nest until they were banded with individually-numbered metal rings on day 6. Nestlings were then raised by either their own parents or unrelated foster parents and they shared the nest environment with both full siblings and unrelated nestlings. For each nestling, growth rates and parasite loads were recorded for the nest and the individual (day 2, 4, 6, and 12). Nestling mass was measured using a digital balance to the nearest 0.01g. Parasite counts were done for individuals by combing through developing feathers and carefully examining the body for mites. Mites were counted in the containers used to hold nestlings and nest mite loads were estimated by the researcher placing their hand in the nest for 30 seconds (timed using a digital stopwatch) and then counting the number of mites on the hand after withdrawal (Saino et al. 2002). Blood films, blood samples for paternity analysis, ventral feathers, right wing length, and body mass were taken for each nestling near fledging (day 12).

4.2 Measuring immune response

White blood cell differentials were obtained to assess nestling immune response. On day 12, blood samples were taken by puncture of the brachial vein using a sterile hypodermic needle and heparinized capillary tubes. Blood films were only taken for nestlings born in the second half of the field season in order to control for naturally varying parasite prevalence across the season (Hund et al. unpub). One drop of blood from the capillary tube was immediately used to create a blood film through the standard two-slide wedge procedure (Krams et al. 2012). Blood samples were taken within 1 hour from the start of handling to ensure that H/L ratios did not change due to handling stress (Davis 2005). The films were air-dried and stained using Wright-Giemsa Quick stain (Vinkler et al. 2010). Films were examined with a light microscope (1000x magnification with oil immersion) in areas where the red blood cells had separated into a monolayer. Counts of lymphocytes, heterophils, basophils, monocytes and eosinophils were

taken until 100 leukocytes had been observed; these counts are assumed to be a reliable proxy for circulating white blood cell proportions (Vinkler et al. 2010). Number of thrombocytes per 100 leukocytes was also recorded but was not a part of the white blood cell differential. Blood films were read blind in relation to the experimental treatment (parasite treatment and cross-fostered treatment). H/L ratio was calculated by dividing the proportion of heterophils by the proportion of lymphocytes in each film. There were two readers. Reader 1 read 99 films; they repeated 10 of their own. Reader 2 read 10 slides; they repeated 2 of their own. 7 films were repeated between readers. Repeatability was assessed between readers and within individual readers. There was no significant difference between the H/L ratio, proportions of heterophils and proportions of lymphocytes between readers ($p=0.188$). H/L ratio, proportions of heterophils and proportions of lymphocytes were repeatable within individual readers ($r=0.69, 0.70, 0.71$ respectively).

4.3 Statistical analysis

"JMP Pro 10" and "R" were used for statistical analysis. In total, 109 films were read and used for the analysis. I did not have blood films for all nestlings involved in the second brood cross-foster experiment predominately due to nestling mortality prior to day 12. 66 films were used for the parasite addition treatment ($n=66$ nestlings) and 43 films for the natural treatment ($n=43$ nestlings).

A general linear mixed model was used to test if the experimental addition of mites had lasting effects on the exposure of nestlings to mites. The individual number of mites on day 12 nestlings in relation to experimental treatment was examined. To meet the assumption of linear models, individual mite counts (which tended to be zero inflated) were normalized using a natural log transformation. Moreover, variation due to breeding site and nest effects was controlled for using the random effects 'site' and 'nest identification' in the model.

The H/L ratio was used as an index of immune response in order to determine if ectoparasites elicited an immune response. A general linear mixed model was used to explore whether nestling H/L ratio differ in nestlings exposed to parasites (parasite treatment) and as a function of being cross-fostered (if it was "exchanged" or left in its natal nest). H/L ratios also tended to be zero inflated; the distribution was normalized using a natural log transformation. To avoid problems associated with covariate multicollinearity, the intercorrelations between mite counts, day 12 body mass (nestling body mass, hereafter) and right wing length were analyzed using a Spearman's rank correlation analysis. As nestling body mass was strongly correlated to the length of the right wing ($p < 0.001$) only one of these variables (mass) was used in the multivariate model constructions. Because H/L ratios were correlated with nestling body mass, this variable was retained in the analysis. Once again, variation due to breeding site effects was controlled for using the random effect "site" and "nest identification" in the model. There was no significant relationship between whether a nestling was "exchanged" (during the cross-foster experiment) and H/L ratio or nestling body mass and the variable "exchange" was removed from the analyses.

In the proceeding general linear mixed model, there was a significant interaction between H/L ratio and nestling body mass. To further examine this relationship the effect each individual treatment had on the relationship between nestling body mass and H/L ratio was analyzed. In order to interpret the individual effects of each treatment the data was sub-setted for the parasite addition treatment and the natural treatment and two separate models were run: (1) a general linear mixed model analyzing H/L ratio as a function of nestling body mass in the natural treatment (2) a general linear mixed model analyzing H/L ratio as a function of nestling body

mass in nestlings in the parasite addition treatment. For both model (1) and (2) variation due to breeding site effects was controlled for using the random effect “site” and “nest identification”.

After finding out that the parasite addition treatment did elicit an immune response, the different branches of the immune system were examined in order to tell if the acquired or innate immune response was driving differences in the H/L ratio. In order to do this the effect treatment had on heterophil and lymphocyte proportions was analyzed. The relationship between the proportion of lymphocytes and the proportions of heterophils was then assessed within each individual treatment. Four separate models were run: (1) a general mixed linear model analyzing the relationship between heterophil proportions and treatment (2) a general mixed linear model analyzing the relationship between lymphocyte proportions and treatment (3) a linear model analyzing the proportion of heterophils as a function of lymphocytes within the parasite addition treatment (4) a linear model analyzing the proportion of heterophils as a function of the proportion of lymphocytes within the natural treatment. In order to avoid problems with covariate multicollinearity, the correlations between heterophil proportions, lymphocyte proportions, mite counts, and nestling body mass were analyzed using a Spearman's rank correlation analysis. Nestling body mass was significantly correlated with heterophil proportion so this was retained as a variable in model (1). Variation due to breeding site effects was controlled for using the random effect "site" and "nest identification" in model (1), (2), (3), and (4).

The effects each treatment had on nestling body mass and the acquired and innate immune response was then examined. In order to do this four separate models analyzing the relationship between the proportions of lymphocytes, proportions of heterophils and nestling body mass within each treatment were run: (1) a general linear mixed model analyzing the

relationship between heterophils and nestling body mass within the parasite addition treatment (2) a general linear mixed model analyzing the relationship between heterophils and nestling body mass within the natural treatment (3) a general linear mixed model analyzing the relationship between lymphocytes and nestling body mass within the parasite addition treatment (4) a general linear mixed model analyzing the relationship between lymphocytes and nestling body mass within the natural treatment.

5. Results

5.1 Effectiveness of parasite exposure treatment

Mites loads on nestlings (12 days old) were influenced by parasite manipulation treatment ($F_{1,107} = 4.80$, $p = 0.036$). Least mean square differences indicate that nestlings in parasite addition nests had significantly higher individual mite counts on day 12 than nestlings in natural nests (Fig. 1). This result confirmed the effectiveness of parasite addition experiment and indicates that nestlings in this treatment were exposed to parasites throughout early development.

5.2 Question 1: Do ectoparasites elicit an immune system response in barn swallow nestlings?

H/L ratios were influenced both by the parasite treatment ($F_{1,107} = 8.89$, $p = 0.004$) and nestling body mass (g; $F_{1,107} = 8.72$, $p = 0.004$). H/L ratios were significantly greater in parasite addition nests than in natural nests (Fig. 2). Whether a nestling was exchanged or left in its natal nest was not significantly correlated with nestling mass or H/L ratios, indicating that the increased handling time and colored legs of the swapped nestlings did not impact mass or white blood cell differentials ($F_{1,107} = 0.07$, $p = 0.78$).

5.3 Question 2: Do ectoparasites influence patterns of immune response in barn swallow nestlings?

Heterophil proportions were influenced by both treatment ($F_{1,107}=5.35$, $p = 0.02$) and nestling mass (g; $F_{1,107}=22.5$, $p < 0.001$). The proportion of heterophils was significantly greater in the parasite addition treatment than in the natural treatment (Fig. 3).

Lymphocyte proportions were influenced by treatment ($F_{1,107}= 5.37$, $p = 0.03$). Proportions of lymphocytes were significantly lower in the parasite addition treatment than in the natural treatment (Fig. 4).

In nestlings in the parasite addition treatment and the natural treatment, the proportion of heterophils was inversely associated with the proportion of lymphocytes ($p < 0.001$, $n=66$; $p < 0.001$, $n=43$) (Fig. 5).

5.4 Question 3: Does ectoparasite exposure influence the relationship between nestling immune response and nestling mass?

As stated above, preliminary analysis using a general linear mixed model indicated a negative correlation between nestling mass and H/L ratio across both treatments (g; $F_{1,107} = 8.72$, $p < 0.05$). To examine the specific effects each treatment had on the relationship between nestling body mass and H/L ratio, separate general linear mixed models analyzing H/L ratio and nestling body mass were run for each treatment. H/L ratio was found to be influenced by nestling mass in the parasite addition ($F_{1,65}=21.1$, $p < 0.001$) but was not influenced in the natural treatment ($p=0.91$, $n=43$) (Fig. 6).

The effect of each treatment on the different components of the immune response and nestling mass was further examined by assessing the relationship between nestling mass and heterophil proportions in each treatment. Heterophil proportions were inversely associated with

nestling mass in the parasite addition treatment ($F_{1, 65} = 28.5$, $p < 0.001$) but was not in the natural treatment ($p=0.17$, $n=43$) (Fig. 7).

The effects of treatment on the relationship between the proportion of lymphocytes and nestling body mass were also investigated. Lymphocyte proportions were influenced by nestling mass in the parasite addition treatment ($F_{1, 65} = 10.26$, $p=0.002$) but were not influenced by nestling mass in the natural treatment ($p=0.39$, $n=43$) (Fig. 8). In the parasite addition treatment, as lymphocyte proportions increased nestling mass increased.

6. Discussion

6.1 Question 1: Do ectoparasites elicit an immune response in barn swallow nestlings?

Importantly, the parasite manipulation experiment was successful at increasing mite exposure to the nestlings in the parasite addition treatment group; thus I was able to analyze causal relationships between parasite exposure and the immune system. On average, nestlings in nests inoculated with mites on day 2 had higher mite levels on day 12 than those left under natural nest conditions. This allows me to assume that nestlings in the parasite addition treatment were subjected to significantly higher levels of parasite exposure than those in the natural treatment and allows me to make comparisons between treatments accordingly.

My findings indicate that nestlings in the parasite addition treatment, on average, had higher H/L ratios than those in the natural treatment. While the H/L ratio cannot be used to measure the ability of the immune response, it can indicate that a parasitic infection is generating an immune reaction (Davis et al. 2008, Dufva and Allander 1995, Figuerola et al. 1999). Therefore, the higher H/L ratio in the parasite addition treatment suggests that ectoparasites do elicit an immune response in barn swallow nestlings.

6.2 Question 2: Do ectoparasites influence patterns of immune response?

Nestlings in the parasite addition treatment had, on average, a significantly higher proportion of heterophils [innate leukocyte] and significantly lower proportion of lymphocytes [acquired leukocyte] than nestlings in the natural treatment.

Both branches of the immune system are important in mounting an immune response to ectoparasites (Owen et al. 2009). Varying relationships between heterophil and lymphocyte levels have been found previously in response to parasite exposure in nestlings (Shutler et al. 2010). The relationship here suggests a greater proliferation of heterophils than lymphocytes, a lack of lymphocytes, or a combination of both a rise in the proportion of heterophils and decrease in the proportion of lymphocytes in response to mite exposure.

The significantly higher proportion of heterophils in the parasite addition treatment may indicate that barn swallow nestlings primarily utilize their innate immune response in order to combat ectoparasites (Klasing and Leshchinsky 1999, Szabo et al. 2002, Dehnhard 2011). Nestlings may use a heterophil-based response because it is less costly to develop and more immediately available; however it is more damaging to self-tissue (Blount et al. 2003, Bonneaud et al. 2003, Lee 2006). In turn, the acquired immune system is more targeted and less damaging, yet more expensive and timely to develop (Dehnhard et al. 2011). It is important to note that a stronger innate immune defense may be conducive to short term success (i.e. fledgling success) but a better developed acquired immune defense is more advantageous to long term fitness (Lochmiller and Deerenberg 2000).

One reason that nestlings may predominantly utilize their innate immune response is because the acquired immune system is not yet developed; nestlings may compensate through the proliferation of heterophils (Apanius 1998). The patterns seen here are parallel to other

studies that show that nestlings may depend more on their innate immune system than their acquired immune system during early life stages; however, the costs and benefits associated with each branch of the immune system may explain why studies sampling multiple ages of development have found a similar pattern to mine in younger nestlings but a heavier reliance on the acquired immune system in more developed nestlings and adult birds (Lee 2006, Palacios et al. 2009, Dehnhard et al. 2011)

Another potential explanation for the pattern of increased heterophil proportions and decreased lymphocyte proportions seen here is that nestlings exposed to ectoparasites may be investing in the innate immune system at the detriment of the acquired immune system (Norris and Evans 2000). Nestlings exposed to ectoparasites may have lower proportions of lymphocytes when those not exposed to ectoparasites because they are simultaneously investing in immune defense and somatic development and may have to forgo allocating resources on the costly development of the acquired immune system (Klasing and Leshchinsky 1999, Lee 2006). The concept of varying lifetime pressures influencing different components of the immune response and development has been studied across other avian taxa (Blount et al. 2003, Bonneaud et al. 2003, Lee et al. 2006). House sparrows have a better-developed acquired immune system than tree sparrows, while tree sparrows have been found to generate a stronger non-specific response (Lee et al. 2006). Since tree sparrows have higher reproductive rates and shorter lifespans a better developed innate immune function has the benefit of allowing tree sparrows to survive until breeding age without compromising early reproductive success by allocating resources towards the development of the acquired immune function (Lochmiller and Deerenberg 2000, Lee et al. 2006). The costs of reproduction are often thought to be analogous to the costs of somatic growth and parasite defense (Lope et al. 1993). Similarly, while it has not yet been

studied within a population, nestlings experiencing more pressure from ectoparasites may utilize their less costly innate immune function than their acquired immune function to avoid sacrificing their somatic growth. The possibility of a trade-off between the innate and acquired immune system is supported by the finding that nestlings with a higher proportion of lymphocytes did not also exhibit a high proportion of heterophils, possibly because once the acquired immune system has developed a strong innate immune system is no longer as important (Dehnhard et al. 2011).

Nestlings that were exposed to parasites during early development had higher proportions of heterophils compared to those in my control group. However, the number of heterophils was dependent on the number of lymphocytes indicating a strong correlation between the two branches of the immune system. The present results indicate that nestlings use their innate immune system to predominately defend against ectoparasites and, potentially, that ectoparasite exposure at an early age may influence the function of the acquired immune response.

Future research should include sampling leukocyte profiles throughout multiple stages of nestling growth in order to track immunological development throughout the nesting period. The relationship between lymphocyte and heterophil proportions could be better resolved through obtaining entire white blood cell counts (Masello et al. 2009); although the latter are more amenable to lab studies versus field studies. Furthermore, in order to clearly identify how ectoparasite exposure at an early age impacts the immune system better measures of the strength of the innate immune response and acquired immune response against natural pathogens in free-living systems must be developed (Norris and Evans 2000).

6.3 Question 3: Does ectoparasite exposure influence the relationship between the immune response of nestlings and mass?

I used mass as an index of size throughout my study due to the tight correlation between right wing length and nestling body mass. I found that the H/L ratio of nestlings in the parasite addition treatment increased as mass decreased and that there was no significant relationship between H/L ratio and mass in nestlings in the natural treatment. Primarily, these findings suggest that the environmental stress associated with resource availability, and, therefore, smaller mass does not directly correlate with H/L ratios in my sample population (Suorsa et al. 2004). Therefore, the relationship between H/L ratio and mass is driven by ectoparasite exposure. Previous authors have claimed that small body mass in relation to the H/L ratio is an indicator of reduced immune efficacy (Krams et al. 2012). However, because the ideal immune response of nestlings to ectoparasites has yet to be defined, these findings cannot unequivocally be taken as a sign of immunosuppression (Norris and Evans 2000, Davis et al. 2008). My study does coincide with other studies that claim higher H/L ratio and lower body mass is the result of having to divide limited resources between energetically demanding activities (i.e. parasite defense and growth indicated by body size) (Saino et al. 1998, Quillfeldt et al. 2008).

Furthermore, it has been suggested that resource availability (represented by body size) may influence the different branches of the immune system in varying ways (Norris and Evans 2000). My study found that the proportion of heterophils in nestlings in the parasite addition treatment had a negative correlation with nestling body mass. Furthermore, the proportion of lymphocytes in the parasite addition treatment had a positive correlation with mass. The development of lymphocyte-mediated responses is believed to be the most sensitive to resource availability (Apanius 1998, Klasing 2004). My study reflects these conclusions; nestlings of

higher mass could better afford to produce lymphocytes and did not rely as heavily on heterophils (Figure 7, Figure 8). The varying relationships between mass and either branch of the immune system within the parasite treatment better supports the hypothesis that there may be some trade-off between body size (an indicator of growth) and immune response (Lee 2006). Lee (2006) concluded that individuals of smaller size would most likely better develop an innate immune response as opposed to an acquired immune response. Under conditions of low energy resource availability (small mass) combined with pressure from parasites as well as to develop and fledge before being predated, the optimal (not to get confused with maximum) immune response may be a higher investment in the innate immune response (Remes and Martin 2002, Zuk and Stoehrer 2002, Lee 2006).

Bonneaud et al. (2011) highlighted the expense of immune defense by demonstrating that adult females experienced a trade-off between immune response and reproduction (a cost comparable to growth) when subjected to a pathogen. The lack of a relationship between nestling body mass and lymphocyte and heterophil proportions in the natural treatment of the present study is consistent with the assumption that parasite exposure is costly; immune response only interacted with nestling body mass when the nestling was subjected to parasites.

To fully explore the costs of the development of each branch of the immune system and the interaction with body size better tests of the strength of the innate and acquired immune response against a natural pathogen must be developed (Norris and Evans 2002). However, the findings that lymphocyte proportions are more dependent on high body mass is a significant indicator that lymphocyte proportions are more resource dependent. However, future studies are needed in order to determine the full extent of the cost of each branch of the immune system and parasite exposure.

6.4 Conclusion

Ectoparasites were found to elicit an immune response in barn swallow nestlings on day 12 during early development. Nestlings tended to predominately use their innate immune response when exposed to ectoparasites, as indicated by higher proportions of heterophils in the parasite addition treatment. However, the use of either the innate immune response or acquired immune response to defend against ectoparasites was also dictated by nestling mass. This relationship better supports what other studies have proposed: the acquired immune system may be more costly for nestlings to use due to the developmental process involved (Klasing and Leschinsky 1999, Norris and Evans 2002, Lee 2006).

Furthermore, the correlations between nestling size and leukocyte profiles indicate that there is a significant interaction between immune response and nestling mass, which may translate into a trade-off between somatic growth and immune development. In order to better determine the exact trade-off between somatic growth and immune development in the barn swallow study system, future research should involve sampling immune response and body size throughout multiple stages of development in order to understand how leukocyte profiles (and investment in either branch of the immune system) change throughout time in response to ectoparasites and growth.

If ectoparasites do influence somatic growth and immune system development, parasite exposure during development could have broader implications for lifetime fitness. While it has not been studied in birds, it has been found in other vertebrates that exposure to a pathogen during development can influence the range of the immune system response later on in life (Ardia et al. 2011). Impeded somatic growth has also been shown to negatively correlate with recruitment of an individual into the breeding population and decreased reproductive success

(Legge et al. 2002, Donadio et al. 2012, Monticelli and Ramos 2012). Therefore, it is crucial to fully elucidate the influence of ectoparasites on juveniles.

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Tables

Table 1. General overview of the avian immune system

Branch of the Immune System	Function/description	Associated White Blood Cell(s)
Innate Immune System	<ul style="list-style-type: none"> -First line of defense against a foreign invader or substance -Rapid (available even when no immune challenge is present) -Non-specific (does not recognize threat) -Causes tissue damage -Not costly to develop 	<ul style="list-style-type: none"> -granulocytes (heterophil, eosinophil, basophil) -monocyte
Acquired Immune System	<ul style="list-style-type: none"> - Specific (is able to specifically recognize a threat and mount a response accordingly) -Powerful - Only available to an immune challenge following specific antigen signaling - Memory cells enable a fast and targeted response during a second infection; may provide lifetime immunity to a pathogen -Costly to develop 	<ul style="list-style-type: none"> - lymphocytes (T and B cells)

Table 2. Questions with hypotheses and predictions that form the core of this study.

Question	Hypothesis	Prediction
1) Does ectoparasitism elicit an immune response?	1) Ectoparasites do elicit an immune response. 2) Ectoparasites do not elicit an immune response.	1) Nestlings exposed to higher levels of parasite exposure will have significantly different leukocyte ratios than those with low/no parasite exposure. 1) Nestlings exposed to higher levels of parasite exposure will not have significantly different leukocyte ratios in comparison to nestlings with low/no exposure to parasites.
2) How does the immune system of nestlings respond to ectoparasites?	1) Nestlings do not exhibit a pattern of immune response. 2) Nestlings exhibit a stronger investment in innate immunity when exposed to ectoparasites. 3) Nestlings exhibit a stronger investment in acquired immunity when exposed to ectoparasites.	1) There will not be a significant relationship between innate versus acquired immunity in response to parasitism. 1) Nestlings will have a higher proportion of heterophils to lymphocytes in parasitized nests. 1) Nestlings will have a higher proportion of lymphocytes to heterophils in parasitized nests.
3) Does ectoparasite exposure influence a relationship between immune response and size?	1) There is no relationship between parasite exposure, body size and immune response. 2) There is a relationship between parasite exposure, body size and immune response.	1) Nestling leukocyte proportions, parasite exposure, and mass will not be correlated. 1) Nestling leukocyte proportions, parasite exposure and mass will be correlated.

Table 3. Studies comparing parasite exposure and leukocyte profiles in nestlings

Study System	Parasite Manipulation?	Ectoparasite or endoparasite?	Research Question (s)	Conclusion (s)	Reference
Red rumped swallow, <i>Hirundo daurica</i>	Yes	Ectoparasite	- What are the effects of experimental infection with a generalist ectoparasite on hirundines?	- Nestlings in infected nests had higher rates of mortality and smaller body size. - Nestlings in infected nests had higher levels of heterophils (potentially an immune response to ectoparasites)	(Merino et al. 2001)
House sparrow, <i>Passer domesticus</i>	No	Ectoparasite	-What are the physiological consequences of blood-feeding mites on nestlings? -Are there any general health consequences?	- Higher proportions of heterophils positively correlated with mite load (nestlings invested in an innate immune response) - No measures of body size correlated with mite load (possibly because the study site was an area of high resource availability).	(Szabo et al. 2002)
Eurasian kestrel, <i>Falco tinnunculus</i>	No	Ectoparasite	- What are the correlations between H/L ratios, stress-related hormone levels, and environmental stressors in free-living birds?	- Stress-related hormone levels were not related to H/L ratios. -Environmental stress from ectoparasites does not elicit a hormonal reaction in Eurasian kestrels. - Ectoparasite exposure was correlated with higher H/L ratios (possibly due to an innate immune reaction).	(Müller et al. 2011)
Pied flycatchers, <i>Ficedula hypoleuca</i>	No	Endoparasite and Ectoparasite	-What haematological variables (i.e relative leukocyte counts) are affected by body size, parasitism, hatch date and brood size?	- Higher H/L ratios were found in nestlings with higher mite loads (possibly due to an innate immune reaction). - Nestlings of lower mass had higher H/L levels. - Nestlings had higher H/L levels than adult birds.	(Lobato et al. 2005)

Study System	Parasite Manipulation?	Ectoparasite or endoparasite?	Research Question (s)	Conclusion (s)	Reference
Eurasian Kestrels, <i>Falco tinnunculus</i>	No	Endoparasite	- What is the difference between innate immune response and acquired immune response to endoparasites in nestlings and adults?	- Adults had higher H/L levels than nestlings, but nestlings had higher overall relative numbers of lymphocytes and heterophils. - H/L ratio was negatively related to body mass in nestlings.	(Parejo and Silva 2009)
Barn swallow, <i>Hirunda rustica</i>	Yes	Ectoparasite	-What are the effects of ectoparasite infestation in morphology and physiology of nestlings? -What are the trade-offs between parasite defense and nestling growth?	- Nestlings that were inoculated with parasites had higher rates of feather growth. -Nestling body mass and tarsus length were negatively correlated with feather growth. - The levels of eosinophils and lymphocytes increased in response to ectoparasite exposure.	(Saino et al. 1998)
Herring gull, <i>Larus argentatus</i>	Yes	Endoparasite	- What is the immune response to the tapeworm, <i>Diphyllbothrium dendriticum</i> ?	- Nestlings infected with the tapeworm had higher leukocyte numbers mainly due to a higher number of mature heterophils.	(Mazur et al. 2007)

Figures

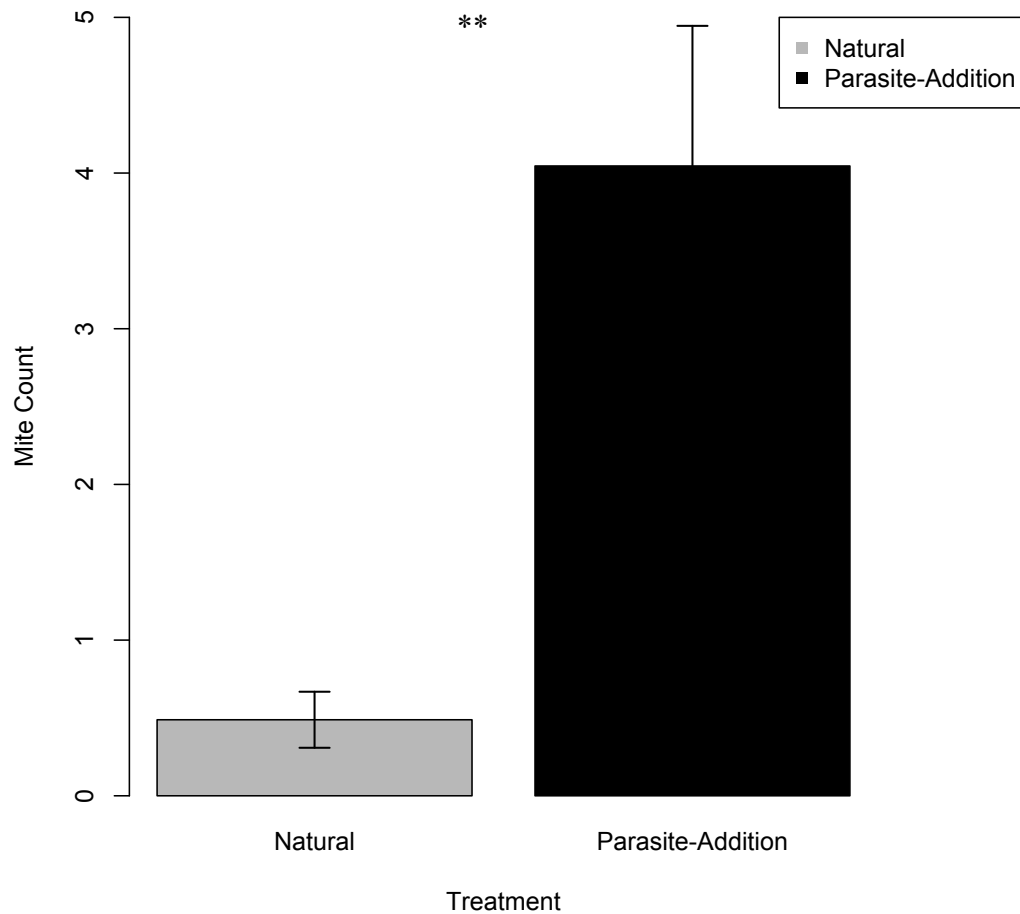


Figure 1. *Mean individual mite count per treatment.* Raw mean of the number of mites found on individual nestlings in the natural treatment (n=43) and the parasite addition treatment (n=66). Mite counts were taken 12 days after hatch date (0) and 10 days after mite inoculation in the parasite addition treatment (parasites were added two days after hatch date (0)).

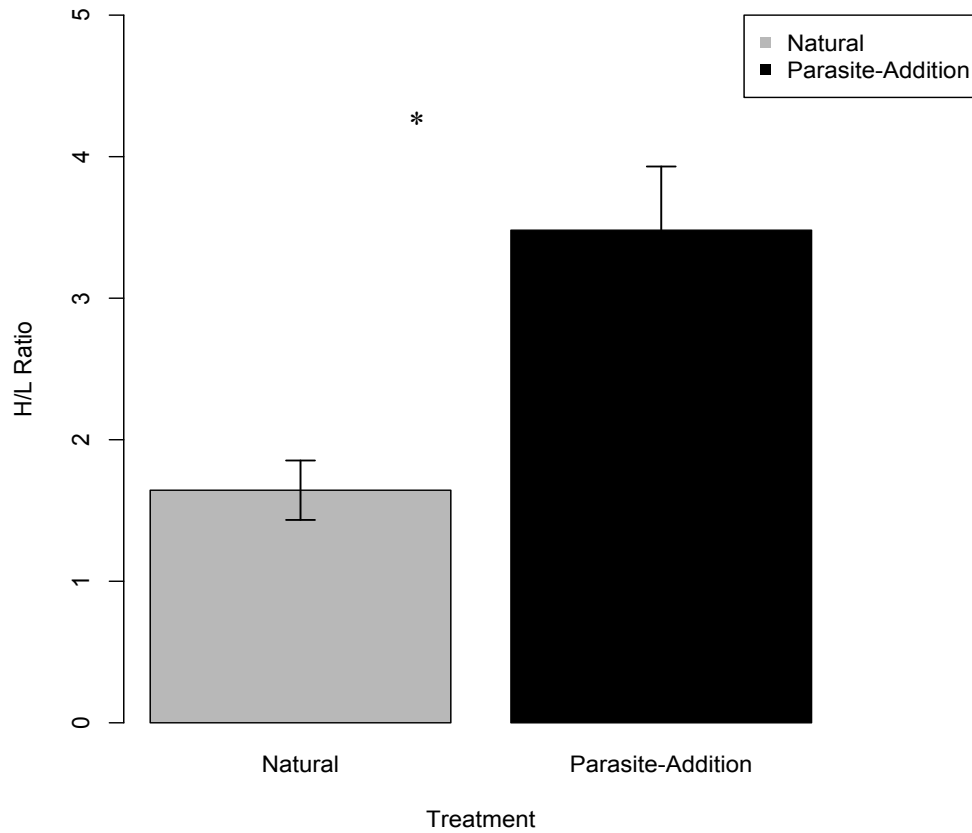


Figure 2. *Mean H/L ratio per treatment.* Raw mean of H/L ratio in the natural treatment (n=43) and the parasite addition treatment (n=66). H/L ratios were based on blood films collected 12 days after hatch date (0) and 10 days after mite inoculation in the parasite addition treatment (parasites were added two days after hatch date (0)).

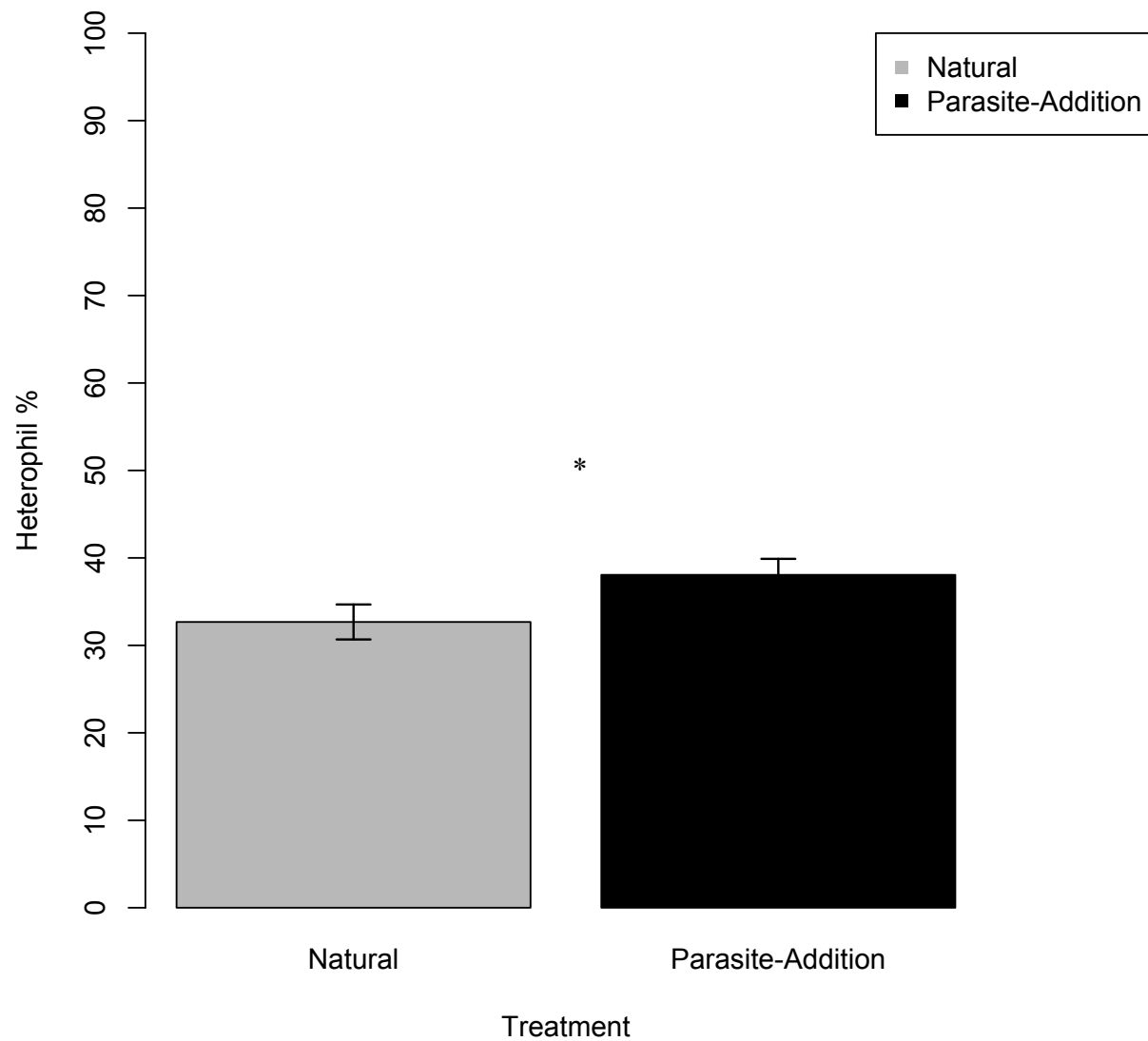


Figure 3. *Mean proportion of heterophils per treatment.* Raw mean of the proportion of circulating white blood cells that are heterophils in nestlings in the natural treatment (n=43) and the parasite addition treatment (n=66). The proportion of heterophils was calculated using a blood film obtained from each nestling 12 days after hatch date (0).

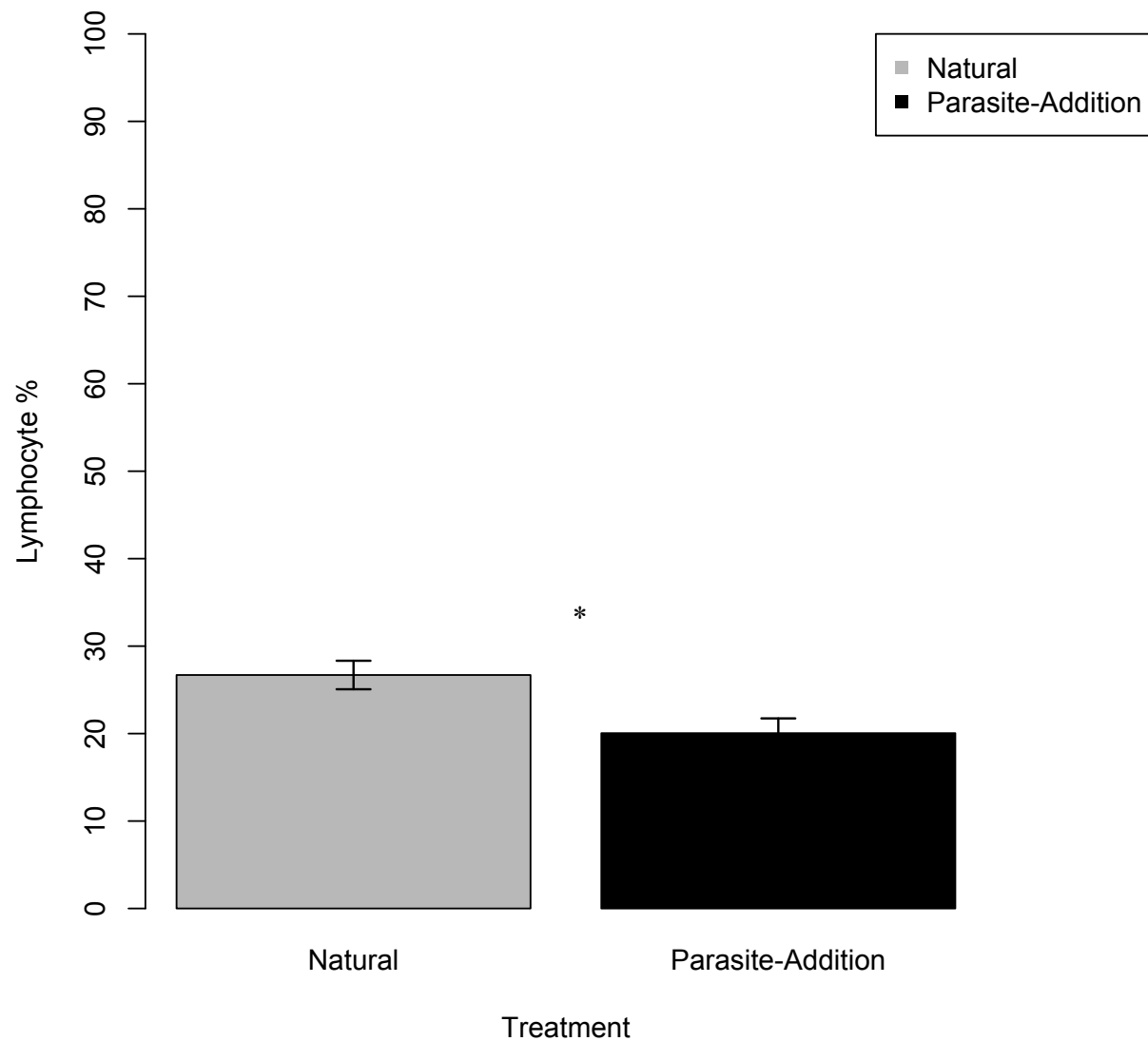


Figure 4. *Mean proportion of lymphocytes per treatment.* Raw mean of the proportion of circulating white blood cells that are lymphocytes in nestlings in the natural treatment (n=43) and the parasite addition treatment (n=66). The proportion of lymphocytes was calculated using a blood film obtained from each nestling 12 days after hatch date (0).

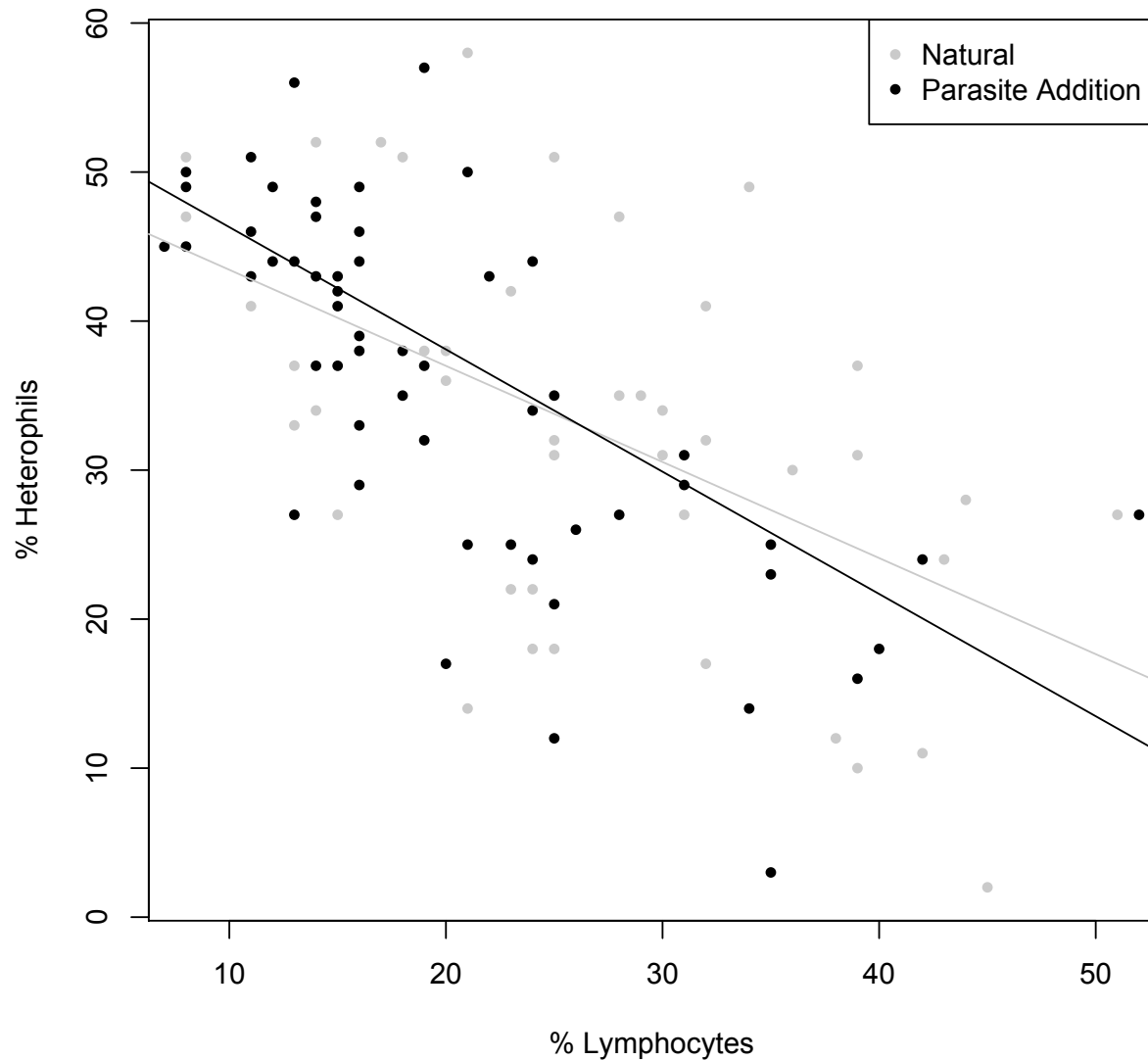


Figure 5. *The proportion of heterophils as a function of the proportion of lymphocytes.* The relationship between the proportion of circulating white blood cells that are heterophils and the proportion of circulating white blood cells that are lymphocytes in nestlings in the natural treatment (n=43) and nestlings in the parasite addition treatment (n=66). Both heterophil and lymphocyte proportions were calculated using blood films obtained from each nestling 12 days after hatch date (0).

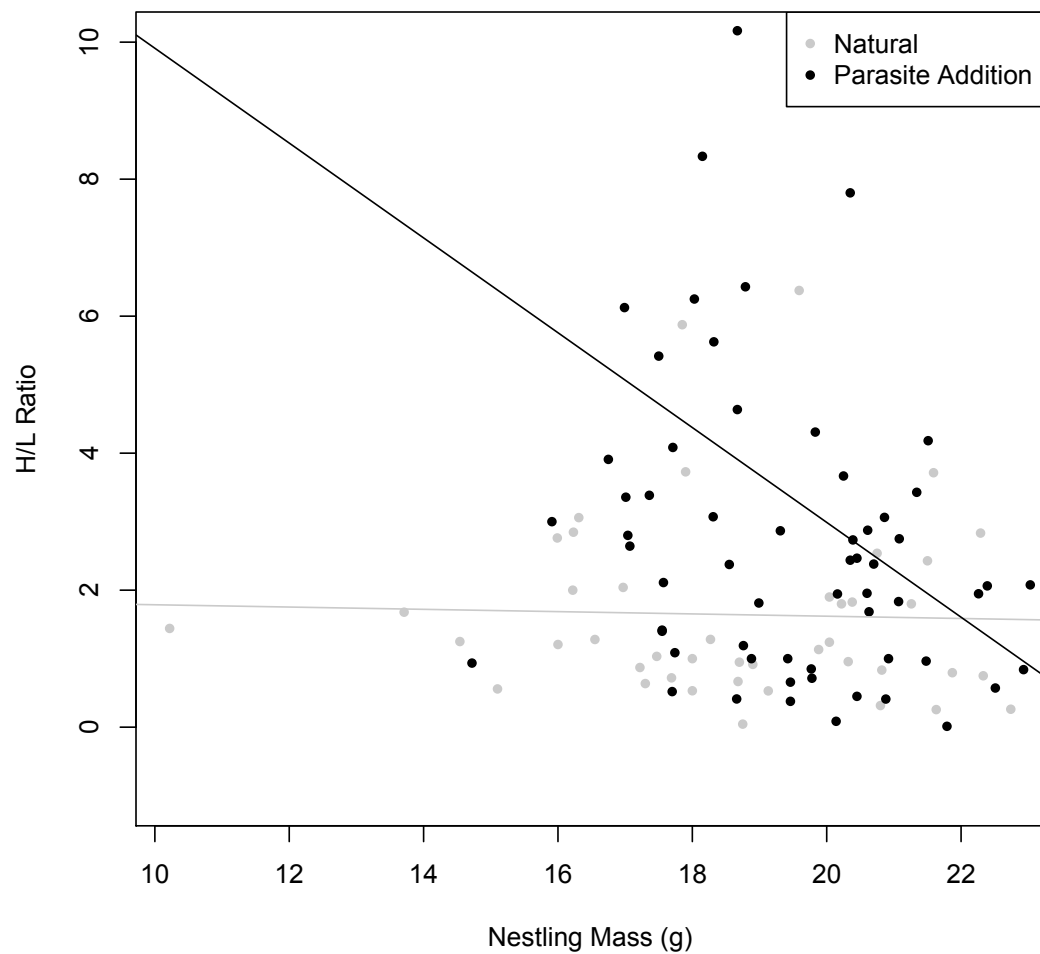


Figure 6. *H/L Ratio as a function of nestling mass (g).* H/L ratio in relation to mass in nestlings in the natural treatment (n=43) and nestlings in the parasite addition treatment (n=66). H/L ratio was calculated using blood films obtained from each nestling 12 days after hatch date (0); mass was measured using a digital scale to the 0.01g 12 days after hatch date (0). The lines represent linear regression lines using the raw data.

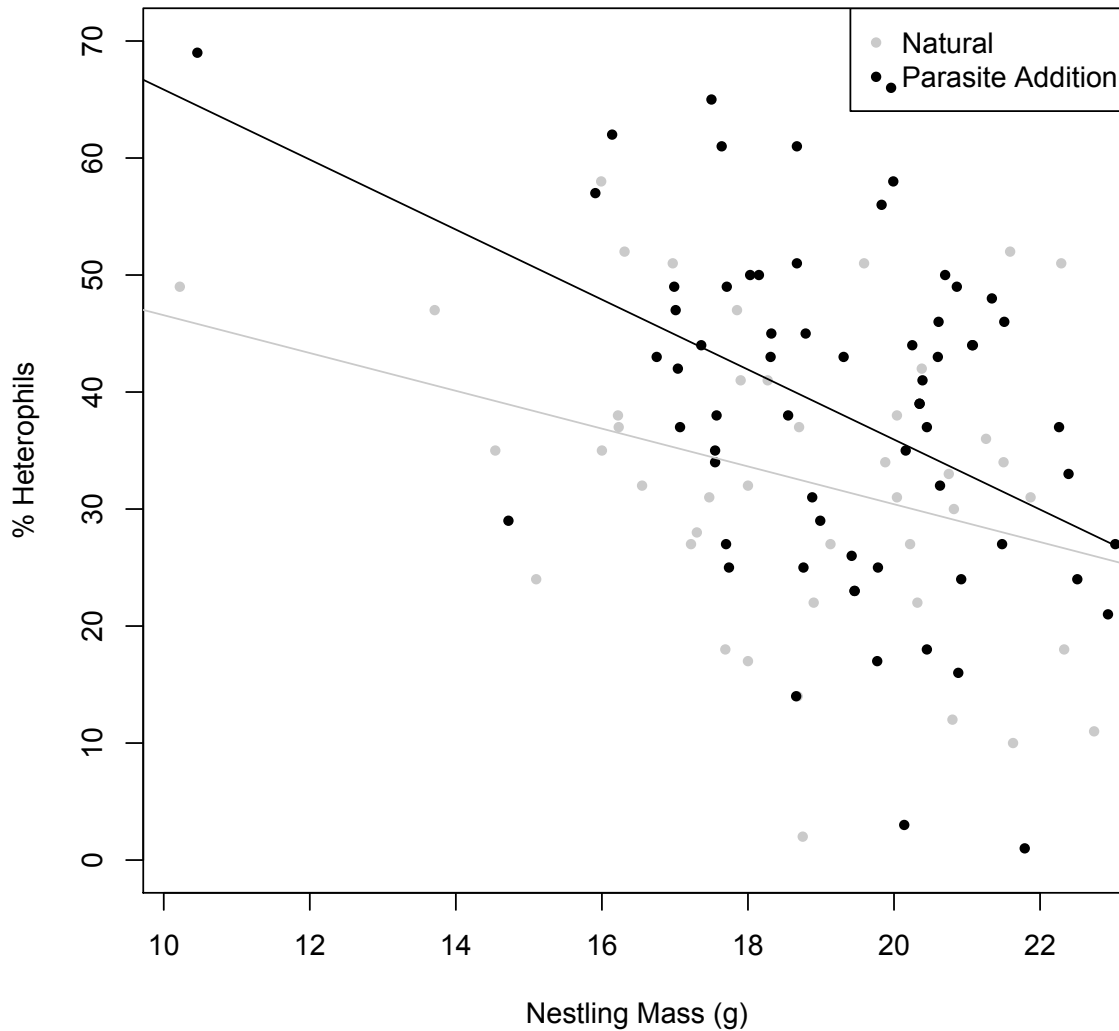


Figure 7. *Proportion of heterophils as a function of nestling mass (g).* The percentage of circulating white blood cells that are heterophils in relation to nestling mass in nestlings in the natural treatment (n=43) and nestlings in the parasite addition treatment (n=66). The proportion of heterophils was calculated using a blood film obtained from each nestling 12 days after hatch date (0); mass was measured using a digital scale to the 0.01g 12 days after hatch date (0). The lines represent linear regression lines of the raw data.

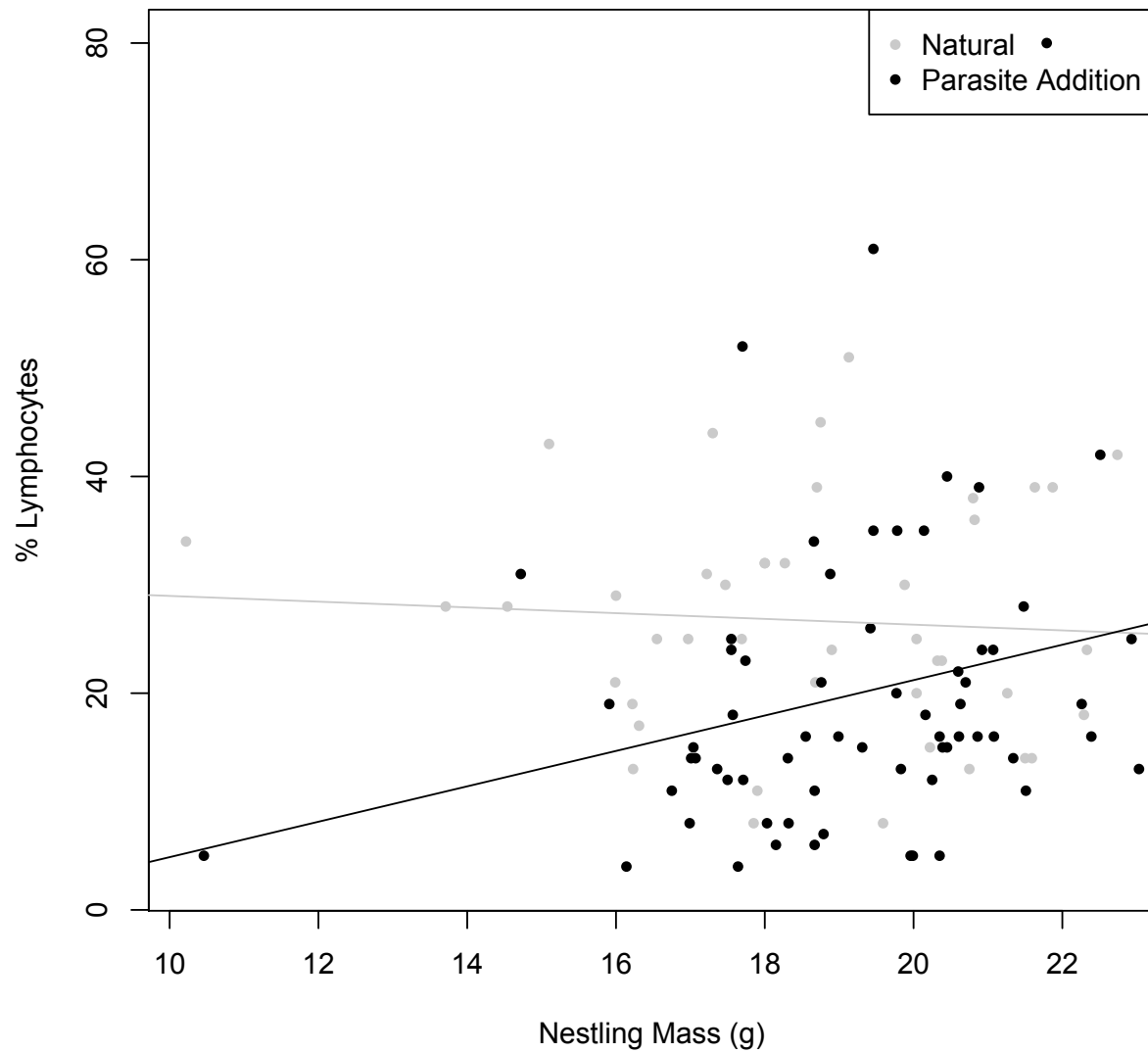


Figure 8. *Proportion of lymphocytes as a function of nestling mass (g).* The percentage of circulating white blood cells that are lymphocytes in relation to nestling mass in nestlings in the natural treatment (n=43) and nestlings in the parasite addition treatment (n=66). The proportion of lymphocytes was calculated using a blood film obtained from each nestling 12 days after hatch date (0); mass was measured using a digital scale to the 0.01g 12 days after hatch date (0). The lines represent linear regression lines of the raw data.

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